



Immunophenotyping and cytokine gene expression in experimental intramammary infection with staphylococcal species in mice*

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ABSTRACT

Immunophenotyping and proinflammatory cytokine gene expression in mice mammary gland inoculated with *Staphylococcus epidermidis*, *S. chromogenes*, *S. haemolyticus* and *S. aureus* isolated from bovine milk were studied. Swiss albino mice were inoculated with 50 µl (2×10⁴ CFU organisms) per mammary gland (L4, L5, R4 and R5) through intramammary route. Mice were sacrificed at 6, 12, 24, 48, 72 and 96 h, and blood and mammary gland tissues were collected for blood immunophenotyping and cytokine gene expression studies, respectively. CD4+ cells increased in *S. aureus*, *S. epidermidis*, *S. haemolyticus* and decreased in *S. chromogenes* inoculated mice. CD8 + cells increased in *S. epidermidis* compared to other groups. Proinflammatory cytokines showed increased expression of IL-1β (6 folds), IL-4 (3 folds), IL-6 (1 fold), IL-12 (5 folds), TNF-α (5 folds) and IFN-γ (2 folds) in *S. aureus* inoculated mice. *S. epidermidis* revealed 2 fold increase in IL-1β and IL-4, and 1 fold increase in IL-6, IL-12, TNF-α and IFN-γ. *S. chromogenes* showed increased expression of 2 folds in IL-1β, IFN-γ; 3 folds in IL-4, IL-6, TNF-α and 7 folds in IL-12. *S. haemolyticus* revealed 2 folds increase in IL-1β, IL-12, TNF-α; 3 folds in IL-6 and 5 folds in IL-4. Hence, *S. aureus* caused severe mastitis in mice when compared to three coagulase negative staphylococcal (CNS) species. The proinflammatory cytokines (IL-1β, IL-4 and IFN-γ) can be used as an indicator for early detection and mice can be used as mastitis model to study CNS mastitis.

Key words: Cytokine, Immunophenotyping, Intramammary infection, Mastitis, Mice, Staphylococcal species

Mastitis is regarded as one of the costliest disease confronting the dairy industry and accounts for 70% of all avoidable losses incurred during milk production (Sadana 2006). Among the bacteria isolated in bovine mastitis, *Staphylococcus* species occupy an important place in dairy animals in India. The meta-analysis of 45 studies reported during 2005–16 revealed pooled prevalence estimates of 45%, 13% and 14% in *Staphylococcus* (*S.*) species, *Streptococcus* species and *Escherichia coli*, respectively from mastitis cases, and period wise prevalence reported to be increasing over the past years for *Staphylococcus* species compared to other major mastitis pathogens (Krishnamoorthy *et al.* 2017a). Common coagulase negative staphylococcal (CNS) species, isolated from bovine intramammary infections routinely, are *S. chromogenes*, *S. simulans*, *S. hyicus* and *S. epidermidis* (Thorberg *et al.* 2009). Prevalence of CNS species was 6–72% and 6–30% in subclinical and clinical mastitis cases, respectively

(Krishnamoorthy *et al.* 2016a). CNS species have traditionally been considered to be minor mastitis pathogens, especially in comparison with major pathogens such as *Staphylococcus aureus*, streptococci and coliforms.

The study of mastitis in bovines is costly and involves various ethical and social issues, especially in India. Moreover, keeping the bovines in controlled environment is difficult. Hence, bovine mastitis is mostly studied in laboratory animal models like mice, rat and rabbit. Previous studies suggested that studying the host response of CNS species in experimental mastitis model is necessary to understand the host pathogen interaction (Simojoki *et al.* 2009, Krishnamoorthy *et al.* 2016a). The mouse mastitis model is regarded as straight forward and suitable model for the study of bovine mastitis which provides valuable information about pathogenic mechanisms of variety of organisms involved in the intramammary infections (Notebaert and Meyer 2006). Scanty information is available on experimentally induced mastitis in animal models by using CNS species especially in mice. Hence, the present study was undertaken to investigate the immunophenotypic and proinflammatory cytokines gene expression in Mice Mammary gland inoculated with three CNS species namely *S. epidermidis*, *S. chromogenes* and *S. haemolyticus*, and one coagulase positive *Staphylococcus aureus*.

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MATERIALS AND METHODS

Three coagulase negative staphylococcal (CNS) species namely *S. epidermidis*, *S. chromogenes* and *S. haemolyticus* and one coagulase positive *S. aureus* were isolated from milk samples of apparently healthy bovines from dairy farms in Karnataka and species identification was carried out as reported earlier (Shome *et al.* 2011, 2012). The timed pregnant (Day 12 to 15), 168 Swiss albino mice were procured from National Centre for Laboratory Animal Science, Hyderabad. The mice were grouped in to 4 groups consisting of 42 mice (36 for bacterial inoculation and 6 for PBS inoculation) with 6 mice in each time points (6 time points) and for 4 organisms (3 CNS and *S. aureus*). The mice were housed in individually ventilated cages, and temperature and humidity of the animal room were maintained at $23\pm3^{\circ}\text{C}$ and 50–70 respectively. Mice were maintained under standard laboratory hygienic conditions, and provided standard laboratory animal pellet feed and reverse osmosis purified water *ad lib*. The animal experiment was approved by Institutional Animal Ethics Committee (IAEC) of Veterinary College, Bengaluru with CPCSEA Registration No.493/01/a/CPCSEA. The experiment was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests and Climate Change, New Delhi. The organisms were inoculated on day 7th or 9th of lactation with dose containing 2×10^4 CFU of bacterial suspension in PBS (50 μl) per teat of left fourth and fifth (L4, L5), and right fourth and fifth (R4, R5) teats. Control mice were inoculated with sterile PBS by intramammary inoculation (IMI) as reported in our earlier studies (Krishnamoorthy *et al.* 2014a, 2014b). After 1 h of inoculation, the pups were allowed to suckle the teat to simulate the natural field conditions of bovines in dairy farms. The mice (from both bacteria inoculated and PBS control groups) were sacrificed at 6, 12, 24, 48, 72 and 96 h.

Blood was collected from intracardiac route into the dipotassiummethylenediaminetetraacetate tubes. The T-lymphocyte subsets were distinguished using dual antibody analysis namely anti-mouse CD4 and CD8 antibodies as described previously (Miao *et al.* 2007) with slight modifications. CD4+ and CD8+ T-lymphocytes in blood were determined using flowcytometry. For immunofluorescence, 10,000 events of each sample were captured. Data acquisition and post-acquisition analysis was conducted by using FACS DIVA software. The CD4/CD8 ratio was calculated by dividing the percentage of CD4+ cells by the percentage of CD8+ cells. The RNA extraction was carried out from mammary gland tissues by using RNeasy mini kit. The RNA concentration was measured using NanoDrop. The cytokine genes, viz. interleukin (IL)-1 β , IL-4, IL-6, IL-12, tumour necrosis factor (TNF)- α and interferon (IFN)- γ expression study was carried in the Real time PCR by Taqman probe method. The primers and probes were designed and used as reported earlier (Modak *et al.*

2012, Krishnamoorthy *et al.* 2016b, 2017b). The fold change in expression of proinflammatory cytokine genes was carried by using $2^{-\Delta\Delta C_T}$ method as described earlier (Livak and Schmittgen 2001). The fold change was expressed as the gene expression in the infected mice over and above the control mice mammary gland. The data obtained were analyzed using Statistical Analysis System (SAS) software version 9.3 (SAS India Limited, Mumbai) by using one way analysis of variance (ANOVA) method (Snedecor and Cochran 1989) and obtained the significant difference between different groups and time points. The results were expressed as the Mean $\pm$ SE (Standard error) with significant difference at $P<0.05$ and confidence interval at 95% level.

RESULTS AND DISCUSSION

The mean percentage of CD4+ cells was 19.16 ± 0.48 , 25.53 ± 1.45 , 16.98 ± 0.72 , 27.82 ± 2.16 and 27.02 ± 0.75 in PBS, *S. epidermidis*, *S. chromogenes*, *S. haemolyticus* and *S. aureus* inoculated mice, respectively (Fig. 1A). The overall mean CD4+ T lymphocytes showed increasing trend in *S. epidermidis*, *S. haemolyticus* and *S. aureus* at different time points which concurred with previous study (Soltys and Quinn 1999), while decreasing trend in *S. chromogenes* infected mice was observed. CD4+ cells reached peak at 24 h after IMI with *S. epidermidis* and *S. haemolyticus*, and at 48 h after IMI with *S. aureus* in mice. However, Miao *et al.* (2007) reported no significant difference between control and 9 h group in T lymphocyte subsets after lipopolysaccharide induced mastitis in rats. *S. chromogenes* showed decrease in the percentage of CD4+ cells when compared to the PBS control which might be due to variation in response of immune system to the infecting organisms. The mean percentage of CD8+ cells was 4.63 ± 0.08 , 12.99 ± 1.09 , 8.54 ± 0.70 , 6.32 ± 0.38 and 6.08 ± 0.18 in PBS, *S. epidermidis*, *S. chromogenes*, *S. haemolyticus* and *S. aureus* inoculated mice, respectively (Fig. 1B). The overall mean CD8+ cells showed increasing trend at different time points after IMI with 3 CNS species and *S. aureus* in mice. CD8+ cells percentage in the present study corroborated with previous reports (Riollet *et al.* 2001, Mehrzad *et al.* 2005, Miao *et al.* 2007). *S. epidermidis* showed significant ($P<0.05$) increase in percentage of CD8+ cells at 6, 12, 24, 48 and 72 h after IMI in mice which concurred with previous report on lactating ewes (Winter and Colditz 2002). Riollet *et al.* (2001) reported that CD8+ cells were mainly recruited in milk compared to CD4+ cells suggesting that CD8+ cells play an important role in chronic *S. aureus* infection. Azeemullah (2010) reported increase in CD8+ cells in rabbit mammary gland compared to CD4+ cells by immunohistochemistry which concurred with this study. The total mean CD4+:CD8+ cells ratio was 4.16 ± 0.13 , 2.11 ± 0.14 , 2.18 ± 0.20 , 4.41 ± 0.21 , and 4.49 ± 0.14 in PBS, *S. epidermidis*, *S. chromogenes*, *S. haemolyticus* and *S. aureus* inoculated mice, respectively (Fig. 1C). CD4+:CD8+ ratio showed significant ($P<0.05$) decrease at 12, 24 and 48 h after IMI with *S. epidermidis* and *S. chromogenes* in mice and was in agreement with previous

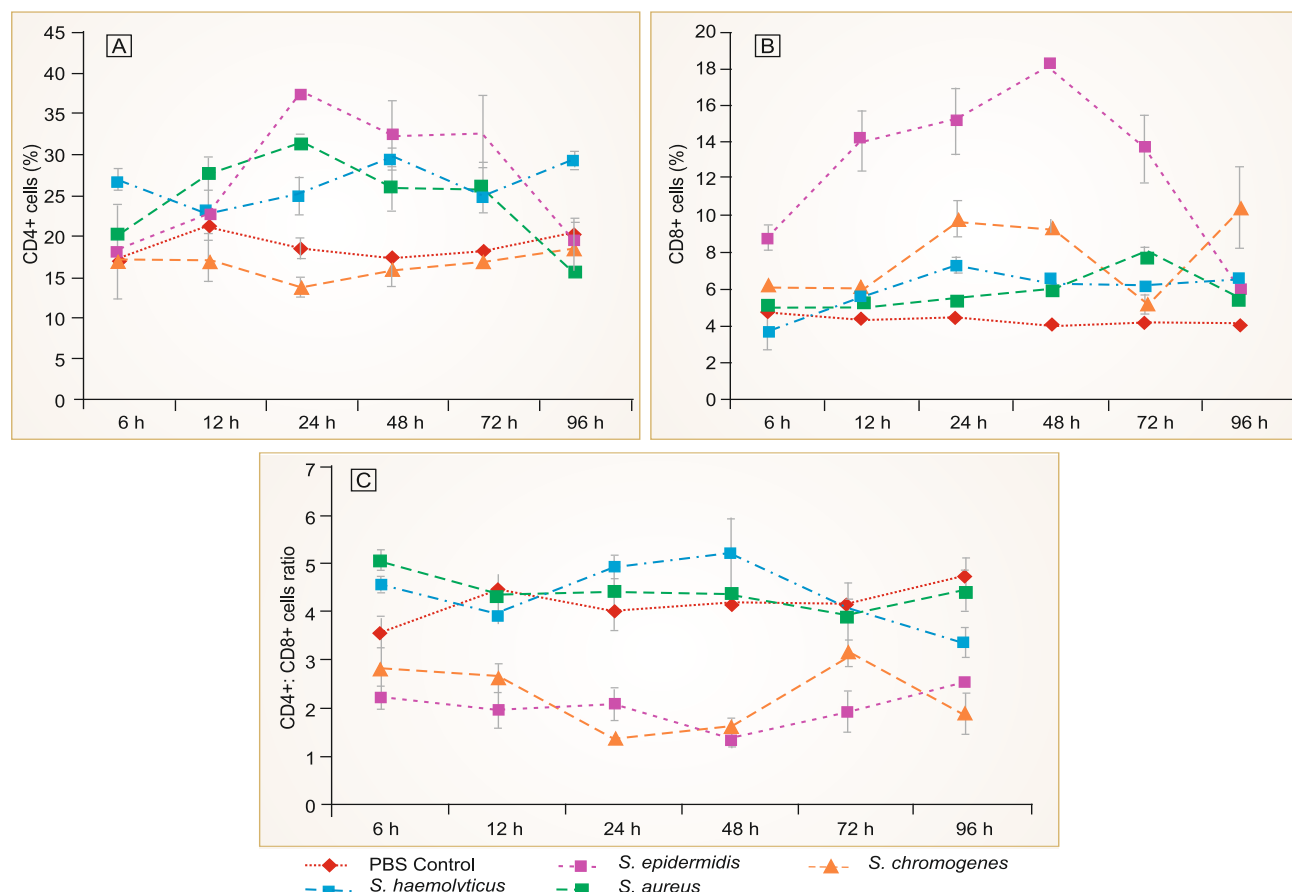


Fig. 1. Immunophenotyping of mice blood inoculated with different staphylococcal species. Percentage of CD4+ cells (A), percentage of CD8+ cells (B) and ratio of CD4+ and CD8+ cells (C).

report (Mehrzhad *et al.* 2005). However, *S. haemolyticus* and *S. aureus* infected mice showed no significant difference in CD4+: CD8+ ratio when compared to PBS control which concurred with previous study (Miao *et al.* 2007). This might be due to the stimulation effect of these microorganisms on T lymphocytes causing an increase in both CD4+ and CD8+ cells in peripheral blood.

The cytokines play an important role in inflammation and act as a mediators of inflammation in host. The mean IL-1 β fold changes were 2.01 ± 0.56 , 1.52 ± 0.33 , 2.50 ± 0.54 and 6.50 ± 2.34 in *S. epidermidis*, *S. chromogenes*, *S. haemolyticus* and *S. aureus* inoculated mice, respectively (Fig. 2A). IL-1 β showed 3–4 folds increase at 24, 48 h and 27 folds at 96 h after IMI with *S. aureus* which concurred with previous reports (Modak *et al.* 2012, Dego *et al.* 2012). *S. epidermidis* infected mice revealed 5 folds increase of IL-1 β gene expression at 72 h which concurred with previous study (Simojoki *et al.* 2011). At 6 and 96 h after IMI with *S. haemolyticus* in mice showed 3–7 folds increase of IL-1 β , respectively and no previous work has been reported for this organism. IL-1 is crucial to the inflammatory process in the mammary gland infused with endotoxin or with natural or experimental coliform mastitis and bovine epithelial cells *in vitro* (Riollet *et al.* 2001, Waller *et al.* 2003). The mean IL-4 fold changes were 1.75 ± 0.71 ,

3.15 ± 0.70 , 5.99 ± 1.98 and 3.88 ± 2.12 in *S. epidermidis*, *S. chromogenes*, *S. haemolyticus* and *S. aureus* inoculated mice, respectively (Fig. 2B). IL-4 showed increased fold changes at 12 h in *S. epidermidis*, at 12 and 96 h in *S. haemolyticus*, at 24, 48 and 96 h in *S. chromogenes* and at 72 and 96 h in *S. aureus* infected mice, which concurred with previous report in *Escherichia (E.) coli* induced mouse mastitis (Modak *et al.* 2012). The increase of IL-4 in CNS species and *S. aureus* induced mice mastitis model was not reported earlier, which is an important finding from this study. Response of IL-4 cytokine in the mice mammary gland is variable based on infecting microorganisms and its role is to suppress the production of IL-1, TNF, chemokines and vascular adhesion molecules (Dinarello 2000). In the present study, *S. epidermidis* showed increased IL-4 fold change which might be due to presence of allergic factors in the bacteria that stimulated the production of this cytokine at 12 h after IMI in mice. The mean IL-6 fold changes were 0.84 ± 0.28 , 3.48 ± 0.83 , 2.66 ± 0.69 and 1.53 ± 0.30 in *S. epidermidis*, *S. chromogenes*, *S. haemolyticus* and *S. aureus* inoculated mice, respectively (Fig. 2C). Increase in IL-6 gene expression observed in this study concurred with previous reports of mastitis induced with various microorganisms (Modak *et al.* 2012, Dego *et al.* 2012). It is widely believed that the cytokines IL-1

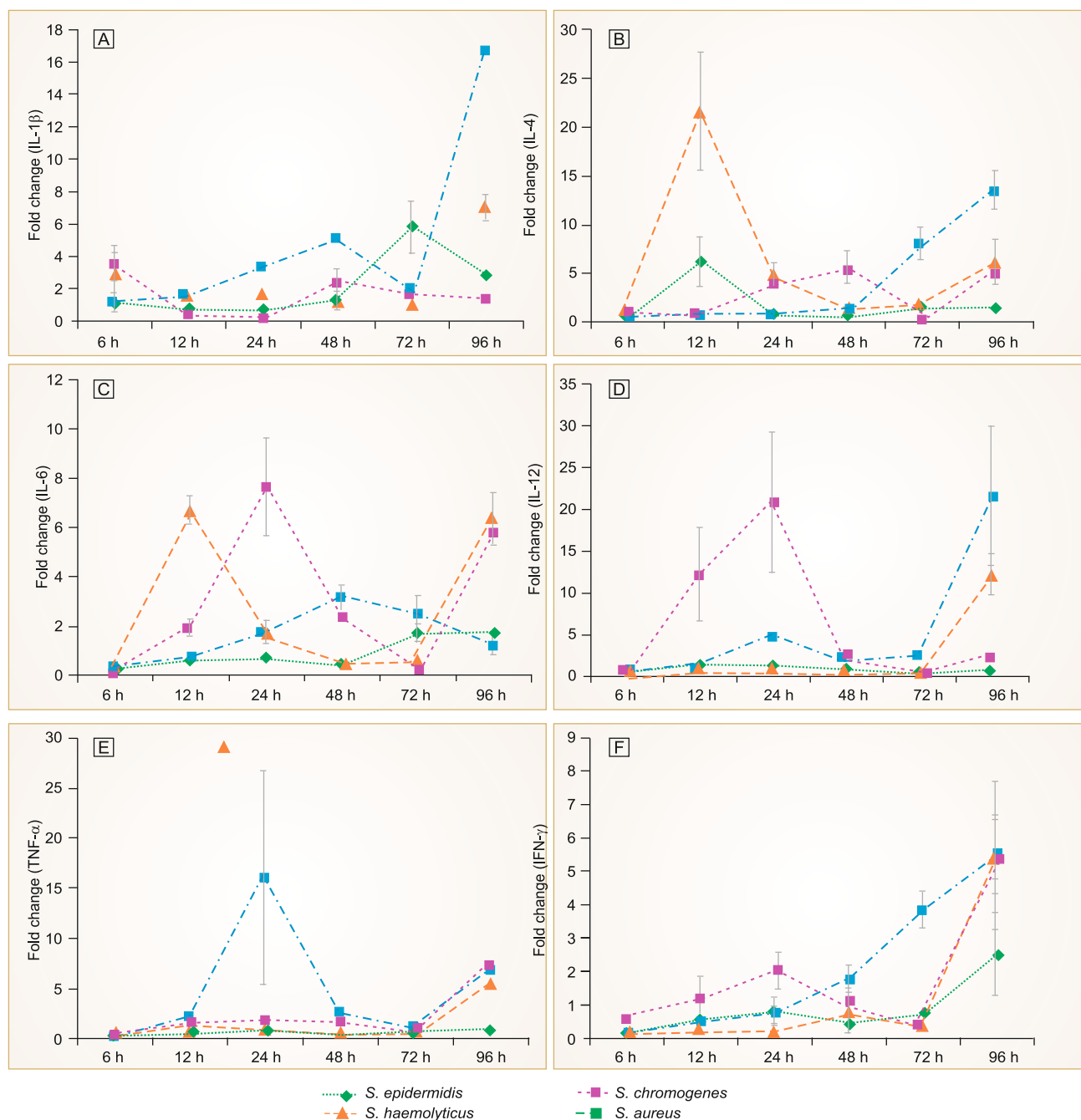


Fig 2. Proinflammatory cytokine gene expression fold changes in mice mammary gland inoculated with different staphylococcal species. IL-1 β (A), IL-4 (B), IL-6 (C), IL-12 (D), TNF- α (E) and IFN- γ (F).

and IL-6 are the main proinflammatory cytokines produced in the host by bacteria and their cell components in inducing mastitis in animals. The mean IL-12 fold changes were 0.78 ± 0.16 , 6.86 ± 2.59 , 2.46 ± 1.11 and 5.22 ± 2.15 in *S. epidermidis*, *S. chromogenes*, *S. haemolyticus* and *S. aureus* inoculated mice, respectively (Fig. 2D). IL-12 gene expression were 12, 20 and 2 folds increased at 12, 24, 48 h, respectively in *S. chromogenes*, 12 folds at 96 h in *S. haemolyticus*, and 21 folds at 96 h in *S. aureus* infected mice which concurred with previous studies with pathogens, namely *S. aureus* (Bannermann *et al.* 2004) and *E. coli* (Modak *et al.* 2012).

The mean TNF- α fold changes were 0.60 ± 0.12 ,

2.56 ± 0.69 , 1.55 ± 0.43 and 4.81 ± 2.00 in *S. epidermidis*, *S. chromogenes*, *S. haemolyticus* and *S. aureus* inoculated mice, respectively (Fig. 2E). TNF- α showed increased expression in *S. aureus* infected mice when compared to three CNS species. Increased TNF- α expression in the mammary gland had been reported after intramammary infection by variety of bacterial pathogens, including *E. coli*, *Streptococcus agalactiae* and *S. aureus* (Chockalingam *et al.* 2005, Bannermann *et al.* 2006). The mean IFN- γ fold changes were 0.72 ± 0.23 , 1.96 ± 0.54 , 1.26 ± 0.51 and 2.06 ± 0.58 in *S. epidermidis*, *S. chromogenes*, *S. haemolyticus* and *S. aureus* inoculated mice, respectively (Fig. 2F). IFN- γ showed 5 folds increased expression at 96

h after IMI with 3 CNS species and *S. aureus* in mice. *S. chromogenes* showed another peak expression at 24 hr and was in agreement with previous reports (Bannerman *et al.* 2004, Kim *et al.* 2001). At the transcript level, increase in IFN- γ mRNA have been detected in cells isolated from the milk of mammary glands infected with *S. aureus* (Riollet *et al.* 2001). IL-12 and IFN- γ are known to induce reciprocal expression, the finding that IL-12 is increased in response to such diverse bacteria is consistent with reports that all these bacteria evoke IFN- γ expression. IL-1 and TNF- α are the major proinflammatory cytokines involved in inflammation, which are locally produced by many cell types and are responsible for early responses. IL-1 and TNF- α triggers an inflammatory cascade and thereby eventually cause fever, inflammation, tissue damage, and in some cases, toxic shock and death. IL-6 is one of the key mediators of the acute phase response in inflammation. The ability of bacteria to establish infection is determined in part, by the nature and rapidity of the corresponding host innate immune response. Therefore the balance between the effects of proinflammatory cytokines is thought to determine the outcome of disease, whether in the short term or long term. Further, the tissue damage in the present study might be perpetuated by all the above mentioned factors and substantially comply with our previous studies (Krishnamoorthy 2014a, 2014b, 2017a, 2017b). The immunophenotyping and cytokine gene expression fold changes indicated that the *S. aureus* caused severe mastitis when compared to other three CNS species.

In the present study, mastitis was successfully induced in mice with different staphylococcal species and mice may be used to study the CNS mastitis in future. The T-lymphocytes subsets mainly CD4⁺ cells increased in *S. epidermidis*, *S. haemolyticus* and *S. aureus*, but decreased in *S. chromogenes* inoculated mice. CD8⁺ cells increased after IMI with *S. epidermidis* in mice. Proinflammatory cytokines may act as potential indicator of CNS species mastitis especially IL-1 β , IL-4 and IFN- γ in mice. CNS species are less pathogenic when compared to *S. aureus* but still it is very important in subclinical mastitis cases in dairy cattle in India.

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REFERENCES

- Azeemulla H R. 2010. 'Pathology of experimental mastitis and studies on the immune responses against *Staphylococcus aureus* biofilm vaccine against mastitis in rabbits'. Ph D Thesis, Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, India.
- Bannerman D D, Paape M J and Chockalingam A. 2006. *Staphylococcus aureus* intramammary infection elicits increased production of transforming growth factor- α , β 1, and β 2. *Veterinary Immunology and Immunopathology* **112**: 309–15.
- Bannerman D D, Paape M J, Goff J P, Kimura K and Lippolis J D. Innate immune response to intramammary infection with *Serratia marcescens* and *Streptococcus uberis*. *BMC Veterinary Research* **35**: 681–700.
- Bansal B K and Gupta D K. 2009. Economic analysis of bovine mastitis in India and Punjab – a review. *Indian Journal of Dairy Science* **62**: 337–45.
- Chockalingam A, Paape M J and Bannerman D D. 2005. Increased milk levels of transforming growth factor- α , β 1 and β 2 during *Escherichia coli* induced mastitis. *Journal of Dairy Science* **88**: 1986–93.
- Dego O K, Oliver S P and Almeida R A. 2012. Host-pathogen gene expression profiles during infection of primary bovine mammary epithelial cells with *Escherichia coli* strains associated with acute or persistent bovine mastitis. *Veterinary Microbiology* **155**: 291–97.
- Dinarello C A. 2000. Proinflammatory cytokines. *Chest* **118**: 503–08.
- Kim Y, Atalla H, Mallard B, Robert C and Karrow N. 2001. Changes in Holstein cow milk and serum protein during intramammary infection with three different strains of *Staphylococcus aureus*. *BMC Veterinary Research* **7**: 51.
- Krishnamoorthy P, Satyanarayana M L, Shome B R and Rahman H. 2014. Mouse milk somatic cell count in coagulase negative staphylococcus species induced mastitis. *Journal of Laboratory Animal Science* **2**(1): 16–19.
- Krishnamoorthy P, Satyanarayana M L, Shome B R and Rahman H. 2014b. Mouse milk bacterial count in coagulase negative *Staphylococcus* species induced mastitis. *Journal of Laboratory Animal Science* **2**(2): 5–8.
- Krishnamoorthy P, Satyanarayana M L and Shome B R. 2016. Coagulase negative staphylococcal species mastitis: an overview. *Research Journal of Veterinary Science* **9**: 1–10.
- Krishnamoorthy P, Sengupta P P, Das S, Ligi M, Shome B R and Rahman H. 2016. Cytokine gene expression and pathology in mice experimentally infected with different isolates of *Trypanosoma evansi*. *Experimental Parasitology* **170**: 168–76.
- Krishnamoorthy P, Suresh K P, Saha S, Govindaraj G, Shome B R and Roy P. 2017. Meta-analysis of prevalence of subclinical and clinical mastitis, major mastitis pathogens in dairy cattle in India. *International Journal of Current Microbiology and Applied Sciences* **6**(3): 1214–34.
- Krishnamoorthy P, Das S, Shome B R and Roy P. 2017. Cytokine gene expression and pathology in experimental *Pasteurella multocida* infection in mice. *Indian Journal of Animal Sciences* **87**(11): 1310–13.
- Livak J K and Schmittgen T D. 2001. Analysis of relative gene expression data using real time quantitative PCR and the 2^{- $\Delta\Delta C_T$} method. *Methods* **25**: 402–08.
- Mehrzaad J, Desrosiers C, Lauzon K, Robitaille G and Zhao X. Proteases involved in mammary tissue damage during endotoxin-induced mastitis in dairy cows. *Journal of Dairy Science* **88**: 211–22.
- Miao J F, Zhu Y M, Gu B B, Wang X B and Zou S X. 2007. Evaluation of the changes of immune cells during lipopolysaccharide induced mastitis in rats. *Cytokine* **40**: 135–43.
- Modak R, Mitra S D, Krishnamoorthy P, Bhat A, Banerjee A and Kundu T. 2012. Histone H3K14 and H4K8 hyperacetylation is associated with *Escherichia coli* induced mastitis in mice. *Epigenetics* **7**: 492–501.

- Notebaert S and Meyer E. 2006. Mouse models to study the pathogenesis and control of bovine mastitis. A review. *Veterinary Quarterly* **28**: 2–13.
- Riollet C, Rainard P and Poutrel B. 2001. Cell subpopulations and cytokine expression in cow milk in response to chronic *Staphylococcus aureus* infection. *Journal of Dairy Science* **84**: 1077–84.
- Sadana V. 2006. Dairy scenario vision 2020, pp. 47–48. *Dairy Year Book*, 3rd edn. Indian Dairy Industry, New Delhi, India.
- Shome B R, Mitra S D, Bhuvana M, Krithiga N, Shome R and Rahman H. 2012. Multiplex PCR for the detection of five important *Staphylococcus* sp. in bovine subclinical mastitis milk. *Indian Journal of Animal Sciences* **82**: 9–14.
- Shome B R, Mitra S D, Bhuvana M, Krithiga N, Velu D and Prabhudas K. 2011. Multiplex PCR assay for species identification of bovine mastitis pathogens. *Journal of Applied Microbiology* **111**: 1349–56.
- Simojoki H, Orro T, Taponen S and Pyorala S. 2009. Host response in bovine mastitis experimentally induced with *Staphylococcus chromogenes*. *Veterinary Microbiology* **134**: 95–99.
- Simojoki H, Salomaki T, Taponen S, Livanainen A and Pyorala S. 2011. Innate immune response in experimentally induced bovine intramammary infection with *Staphylococcus simulans* and *S. epidermidis*. *BMC Veterinary Research* **42**: 49.
- Snedecor G W and Cochran W G. 1989. *Statistical Methods*. 7th edn. Iowa State University Press, Ames, Iowa, USA. pp. 196–252.
- Soltys J and Quinn M T. 1999. Selective recruitment of T-cell subsets to the udder during staphylococcal and streptococcal mastitis: analysis of lymphocyte subsets and adhesion molecule expression. *Infection and Immunity* **67**: 6293.
- Thorberg B M, Danielsson-Tham M L, Emanuelson U and Waller K P. 2009. Bovine subclinical mastitis caused by different types of coagulase negative staphylococci. *Journal of Dairy Science* **92**: 4962–70.
- Waller P K, Colditz I G, Lun S and Ostensson K. 2003. Cytokines in mammary lymph and milk during endotoxin-induced bovine mastitis. *Research in Veterinary Science* **74**: 31–36.
- Winter P and Colditz I G. 2002. Immunological responses of the lactating bovine udder following experimental challenge with *Staphylococcus epidermidis*. *Veterinary Immunology and Immunopathology* **89**: 57–65.